Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 7874–7878, December 1985
Biochemistry

Subunit stoichiometry and juxtaposition of the photosynthetic coupling factor 1: Immunoelectron microscopy using monoclonal antibodies

(spinach chloroplasts/quaternary protein structure/hybridoma technique)

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Communicated by H. Beinert, July 26, 1985

ABSTRACT **Monoclonal antibodies specific to the α subunits of the photosynthetic coupling factor 1 (CF_1) were used as marker molecules in an electron microscopic analysis of the subunit organization of this enzyme. Immune complexes were obtained by incubation of CF_1 with saturating amounts of anti-α-subunit IgG, isolated by gel filtration, and visualized by electron microscopy. The maximum number of antibodies bound to a CF_1 molecule was three, the angle defined by a neighboring pair of antibodies characteristically being 120°. These results are interpreted as direct evidence for the presence of three α subunits in the CF_1 complex, the relative orientation of them being described by 3-fold rotary symmetry. Our observations thus favor an overall subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$.**

Proton translocating ATP-synthases are terminal enzymes of oxidative phosphorylation and photophosphorylation in cell membranes of bacteria, inner membranes of mitochondria, and thylakoid membranes of chloroplasts, respectively. Driven by a transmembrane proton gradient, they catalyze the synthesis of ATP from ADP and P_i (1). While protons are translocated via a membrane-integrated protein complex called F_0, the catalytic sites for ATP formation are located at the F_1 part, an oligomeric protein that can readily be dissociated from the F_0 moiety and then acts as a soluble ATP hydrolyzing enzyme called F_1 ATPase (for reviews, see refs. 2–4).

F_1 ATPases from different sources are similar to each other in that they contain five types of subunits—α, β, γ, δ, and ϵ. However, although there is an increasing interest in the quaternary structure of this class of enzymes, their subunit stoichiometry and arrangement are still not fully understood. Since a number of studies on the functional behavior of F_1 ATPases seem to suggest that two or three catalytic sites, coupled by cooperative interactions, may be involved in enzymatic catalysis (5–7), knowledge of the copy numbers and spatial distribution of subunits is crucial for an understanding of the catalytic process. Hence, efforts to elucidate the subunit stoichiometry of the various F_1 ATPases have been numerous (see refs. 8 and 9 for detailed references).

Although the majority of those working on bacterial and mitochondrial F_1 ATPases now tend to agree on a $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometry (4), the chloroplast coupling factor 1 (CF_1) from thylakoid membranes has long been a prominent candidate for a stoichiometry of $\alpha_2\beta_2\gamma_{1-2}\delta_{1-2}\epsilon_2$ (10–12). Conflicting data are not likely, however, to be the result of actual differences in the enzymes under investigation but rather seem to reflect the different techniques used (8, 9). As discussed in ref. 8, most of these techniques are based on the statistical behavior of interactions between enzyme and reagents. In addition, they rely on an exact determination of the molecular mass. Published values for the molecular mass of CF_1 ATPase, for example, range from 325,000 (10) to more than 400,000 (13), explaining at least part of the contradictory data.

Ambiguities of this kind can be avoided if protein molecules are visualized directly by techniques such as x-ray diffraction analysis or electron microscopy. In the present paper, we report the application of monoclonal antibodies (mAbs) specific to subunits of the CF_1 ATPase from spinach chloroplasts as labels for an electron microscopic study of the protein structure. The rationale of this approach is a direct visualization of the subunits by tagging them with monoclonal IgG molecules (8). Since a mAb can be expected to bind to only one specific epitope per subunit, the maximum number of subunit-specific IgG molecules bound to a CF_1 molecule immediately gives the stoichiometry of that particular type of subunit. At the same time, subunit juxtaposition is revealed by the relative orientation of antibodies bound to CF_1. We found three anti-α-subunit IgG molecules to be bound to one molecule of CF_1 ATPase, the antibodies being orientated in such a way that the angles between them were always close to 120°. These results indicate immediately that three α subunits are symmetrically arranged in the CF_1 complex. Thus, these observations strongly favor an $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometry, at the same time ruling out the $(\alpha\beta\gamma\delta\epsilon)_2$ model.

MATERIALS AND METHODS

Purification of CF_1 ATPase. Thylakoid membranes were prepared according to ref. 14. CF_1 was detached from the membranes as described in ref. 15. After ultracentrifugation (100,000 × *g*, 1 hr), the supernatant was fractionated by gel filtration on a Sephacryl S-300 column equilibrated with 20 mM *N*-tris(hydroxymethyl)methylglycine (Tricine)/2 mM EDTA/1 mM ATP/1 mM dithiothreitol, pH 8.0. Fractions of the second peak contained a latent ATPase that was shown to be homogeneous CF_1 by electron microscopy as well as by polyacrylamide gel electrophoresis (*i*) in the presence of $NaDodSO_4$ (16) and (*ii*) under nondenaturing conditions (6).

Production and Characterization of mAbs. Spleen cells of female BALB/c mice immunized with native CF_1 ATPase were fused with X63-Ag8.653 myeloma cells (17) according to ref. 18. Hybridoma cell lines producing antibodies specific to CF_1 ATPase were cloned under conditions of limiting dilution in a spleen cell-conditioned medium used at 60% strength.

mAbs were concentrated from serum-free culture supernatants, and their immunoglobulin classes were determined by the double diffusion method (19) using IgG- and IgM-

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Abbreviations: CF_1, chloroplast coupling factor 1; mAb, monoclonal antibody; GAM/RAM antibody, goat/rabbit anti-mouse antibody.

specific goat anti-mouse (GAM) antibodies (Nordic, Tilburg, The Netherlands). IgG was purified by affinity chromatography on protein A-Sepharose (20), while pure IgM was obtained after gel filtration on Sephacryl S-300. Binding of purified mAbs to isolated CF_1 was tested using an ELISA (21). Bound mAbs were detected by incubation with an alkaline phosphatase-conjugated GAM (Tago, Burlingame, CA) and subsequent hydrolysis of added *p*-nitrophenyl phosphate. Subunit specificity of the mAbs was analyzed by the immunoblot method (22). mAbs reacting with specific polypeptide bands were decorated with a rabbit anti-mouse (RAM) antibody conjugated with fluorescein isothiocyanate (Tago).

Electron Microscopy. Fifty micrograms of CF_1 ATPase was incubated at 20°C for 10 hr with various amounts of the mAbs in 200 μl of 200 mM Tris·HCl/500 mM glycine, 2% (vol/vol) 1-butanol, pH 7.5. The immune complexes were isolated by gel filtration (23) on a Bio-Gel A-1.5m column equilibrated with 100 mM Tris·HCl/150 mM NaCl, pH 7.5. Samples from fractions containing immune complexes were prepared for electron microscopy directly by negatively staining with 4% (wt/vol) uranyl acetate (24). A Zeiss EM 10 B electron microscope was used at an acceleration voltage of 80 kV, and primary magnifications ranged from 80,000× to 100,000×.

RESULTS

Characterization of mAbs. Antibodies produced by 20 monoclonal hybridomas were analyzed for immunoglobulin class and subunit specificity. mAbs produced by 11 clones were found to bind to the α subunits (immunoglobulin class: 10 IgG, 1 IgM), mAbs produced by two clones reacted with the β subunits (mAbs belonging to the M class), and 7 other clones produced mAbs (IgM) that recognized both the α and the β subunits, and, in two cases, also the γ subunits. No additional polypeptide bands on the nitrocellulose sheet were recognized by any of the mAbs tested when whole chloroplasts were electrophoresed instead of isolated CF_1 ATPase.

Only immunoglobulins of the G class can be used as direct labels for proteins in immunoelectron microscopy; reaction with IgMs would result in the formation of immune clusters too complex to be analyzed in the electron microscope. The capability of monoclonal IgG to bind to isolated native CF_1 ATPase was tested as follows: CF_1 ATPase was incubated with anti-α-subunit IgG for 3 hr; then RAM antibody was added and incubation was continued overnight. The precipitate was spun down, washed, and applied to a NaDodSO$_4$/polyacrylamide gel (Fig. 1). The presence of CF_1 ATPase subunits in the precipitate indicated binding of the respective mAb to the enzyme (lanes 2–4). As a control, two anti-α-subunit IgGs were incubated with F_1 ATPase from beef heart mitochondria. While one of the antibodies did not react with F_1 (lane 6), incubation with the other, apparently because of cross-reactivity, gave an immunoprecipitate containing F_1 ATPase subunits (lane 5). Standards and further controls were electrophoresed in the other lanes (see legend to Fig. 1). As shown in lane 11, an immunoprecipitate containing CF_1 ATPase subunits was formed (although to a smaller extent) even when incubation with RAM antibody was omitted. Since the myeloma cell line chosen for fusion does not produce any immunoglobulin chain by itself, the antibodies produced by the hybrid cells therefore have two identical antigenic binding sites throughout and will form a precipitate with an oligomeric protein containing two or more copies of that type of subunit that exhibits the antigenic determinant.

Immunoelectron Microscopy. In a preliminary experiment, isolated CF_1 ATPase was examined in the electron microscope (Fig. 2 *Inset*). In a number of top view projections, CF_1 ATPase exhibited a hexagonal arrangement of subunits typical for F_1-type ATPases. The single enlarged particle (*Inset*) is shown at a projection angle corresponding to a slightly tilted top view. A seventh protein mass, a characteristic feature also of F_1 ATPases from bacteria (8) and beef heart mitochondria (9), is clearly visible at a central position of the enzyme molecule.

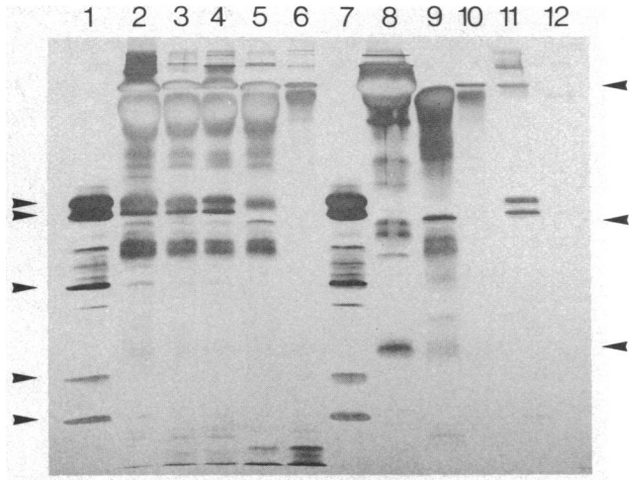

FIG. 1. Binding of native CF_1 ATPase and native F_1 ATPase (beef heart mitochondria) to different monoclonal anti-α-subunit (CF_1) IgGs. Four micrograms of CF_1 (lanes 2–4) or F_1 (lanes 5 and 6) ATPase was incubated for 3 hr with a 10-fold molar excess of IgG [lanes 2 and 5, anti-α (I); lane 3, anti-α (II); lanes 4 and 6, anti-α (III)]. Complexes were precipitated by addition of RAM antibody and incubation for 12 hr. Precipitates were washed twice with phosphate-buffered saline and applied to the gel after denaturation with NaDodSO$_4$ in the absence of sulfhydryl reagents. Binding of monoclonal antibodies to the native enzyme is indicated by bands of CF_1/F_1 subunits (silver stained). Lane 10, 20 μg of anti-α-subunit antibody (I) was incubated with RAM antibody (12 hr); lane 11, 4 μg of CF_1 ATPase was incubated with a 10-fold molar excess of anti-α-subunit antibody (I) (12 hr); lane 12, 4 μg of CF_1 ATPase was incubated with RAM antibody (12 hr). Samples for lanes 10–12 were treated in the same way as those for lanes 2–6. Lanes 1 and 7, 4 μg of CF_1 ATPase was applied to the gel directly; lane 8, 10 μg of anti-α-subunit IgG (I) was applied to the gel directly; lane 9, 10 μg of RAM antibody was applied to the gel directly. CF_1 polypeptides (α, β, γ, δ, ε) are indicated on the left; mAb polypeptides (holo-IgG, heavy chain, light chain) are indicated on the right.

Monoclonal anti-α-subunit IgG (I) was chosen for the first immunoelectron microscopic experiments, while, in later studies, three additional anti-α-subunit IgGs were used. CF_1 ATPase was incubated with an excess of anti-α-subunit IgG at molar ratios ranging from 1:10 to 1:30. Immune complexes were isolated by gel filtration and inspected in the electron microscope. A field micrograph surveying the particle distribution is shown in Fig. 2. Apart from a few free antibodies, one can distinguish one complex made up of one IgG molecule bound to one CF_1 ATPase molecule, another complex composed of three antibodies bound to one CF_1 ATPase molecule, and a number of cyclic complexes composed of two CF_1 ATPase molecules linked by two antibodies. The relative frequencies of the different types of immune complexes shown in this micrograph were not significantly changed when various amounts of ATP were added to the incubation mixtures.

Some examples of the simplest forms of antigen–antibody complexes observed in the electron microscope—complexes of one IgG molecule with one CF_1 ATPase molecule and complexes of two IgG molecules with one CF_1 molecule—are shown in Fig. 3 *a* and *b*. It is important to emphasize that in the latter case the angle defined by the points on the protein surface to which the two antibodies are attached is always close to 120°. This is particularly obvious in Fig. 3*c*, which is

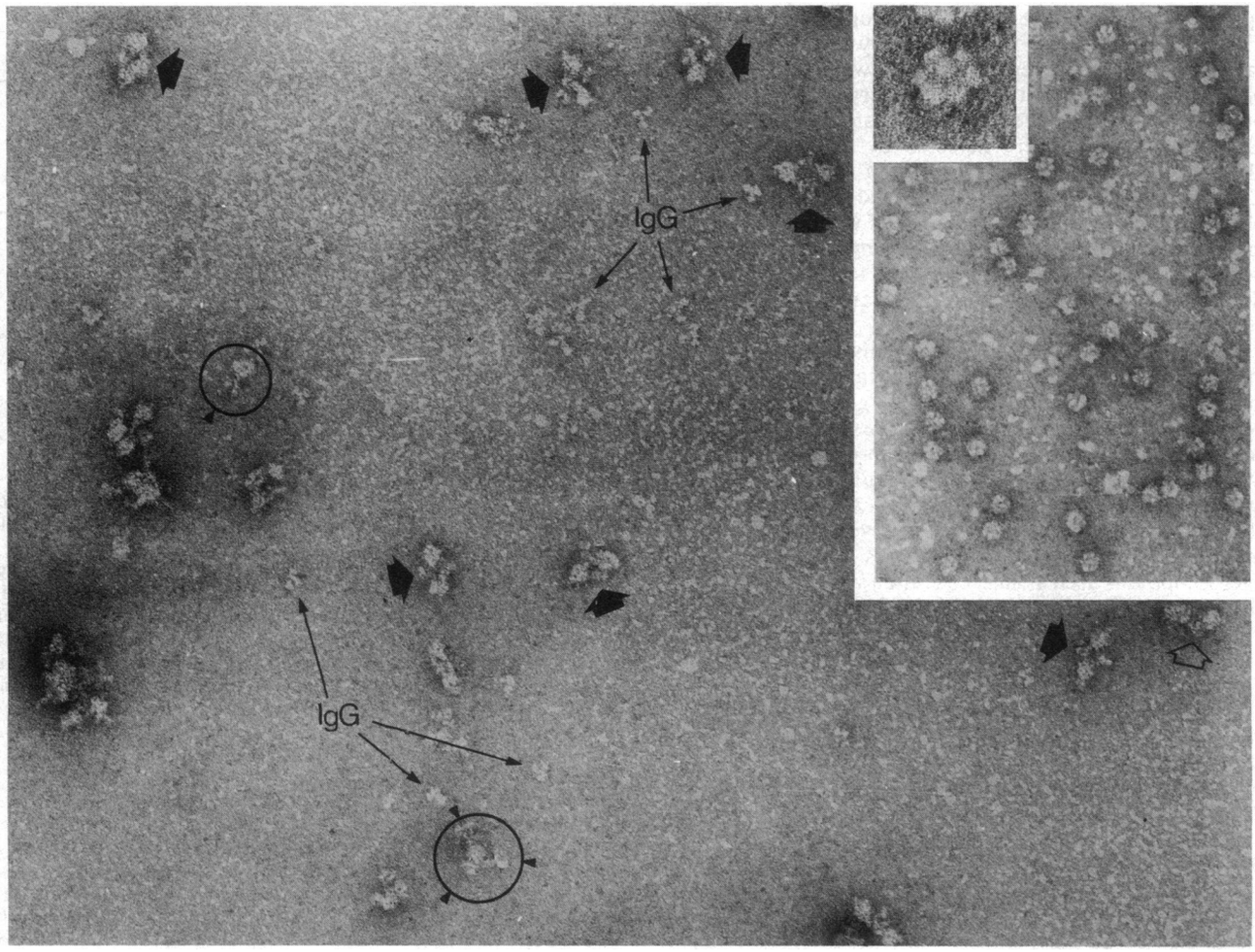

FIG. 2. Electron micrograph of IgG–CF_1 ATPase immune complexes. Anti-α-subunit mAb (I) was used. The different types of immune complexes are identified as follows: encircled, one arrowhead, a complex of one mAb and one CF_1 ATPase molecule; encircled, three arrowheads, a complex of three mAbs and one CF_1 ATPase molecule; solid arrowheads, cyclic complexes of two CF_1 ATPase molecules linked by two mAbs; open arrowhead, a complex of two CF_1 ATPase molecules linked by one antibody. (*Inset*) Electron micrograph of isolated CF_1 ATPase. The maximum diameter of a CF_1 ATPase particle is 12 nm, the center-to-center distance between two peripheral subunits being 4.5 nm. The single enlarged particle is seen at a slightly tilted top view projection. (Bar = 100 nm; single enlarged particle, ×800,000.)

a collection of immune complexes composed of three IgG molecules and one CF_1 ATPase molecule. Here again, the angle between two neighboring antibodies is characteristically 120°, resulting in a 3-fold rotary symmetry of the immune complexes. It should be stressed that the maximum number of antibodies bound per CF_1 ATPase molecule is three. Under no conditions, even when IgG/CF_1 ATPase molar ratios were as high as 30:1, could CF_1 particles be detected labeled with four or more IgG molecules.

The majority of IgG–CF_1 ATPase complexes, however, were of the cyclic type: two CF_1 ATPase molecules linked by two IgG molecules. Examples are shown in Fig. 3*d*. Although the IgG/CF_1 ATPase ratio in these complexes is usually 1, this type was predominant even with CF_1 ATPase that had been incubated with a high excess (30-fold) of IgG, conditions that might be expected to favor formation of immune complexes with higher IgG/CF_1 ATPase ratios. The significance of this observation will be discussed below.

DISCUSSION

F_1-type ATPases form a remarkable class of enzymes in that their subunit stoichiometry and arrangement have been matters of dispute for more than 10 years. Electron microscopy was used rather early to characterize the enzymes from beef heart mitochondria (25) and spinach chloroplasts (26); later it was used to analyze the structure of TF_1, the coupling factor 1 from the thermophilic bacterium PS 3 (27). The quaternary structures of the F_1 ATPases from beef heart mitochondria and *Escherichia coli* have been studied electron microscopically by Tiedge *et al.* (9) and Lünsdorf *et al.* (8), respectively. A common feature of the F_1 particles when observed at a top view projection is a hexagonal arrangement of six peripheral protein masses grouped around a seventh protein mass at the center of a molecule. These and other observations, made while studying the mitochondrial enzyme and corroborated by work on other ATPases of the F_1 type (28, 29), have led to the hypothesis that α and β subunits, three each, are located in different planes at the vertices of equilateral triangles (9), forming an alternating symmetrical sequence of α and β subunits when viewed from above.

In this paper, we report that CF_1, the subunit structure of which has been believed to be "dimeric" (10, 11), is adequately described by the model proposed in ref. 9. Evidence leading to this conclusion may be summarized as follows. (*i*) Electron microscopic studies show that a maximum of three monoclonal anti-α-subunit IgG molecules can bind to one

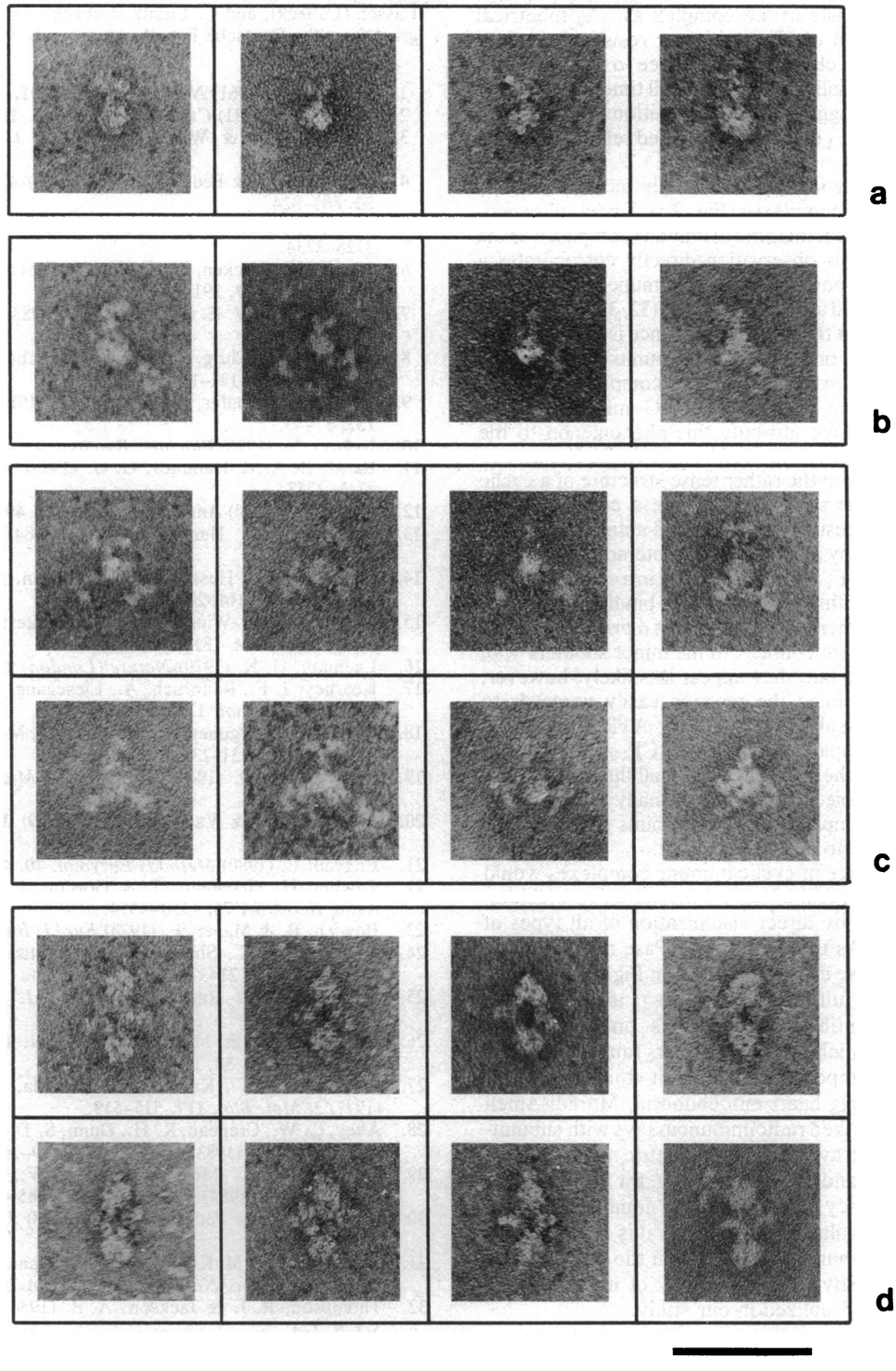

FIG. 3. Four types of (anti-α-subunit IgG)–CF_1 ATPase immune complexes: *a*, one mAb per CF_1 ATPase molecule; *b*, two mAbs per CF_1 ATPase molecule; *c*, three mAbs per CF_1 ATPase molecule; *d*, cyclic complexes containing two CF_1 ATPase molecules, two linking mAbs and, in one case, one nonlinking mAb. (Bar = 50 nm.)

molecule of CF_1 ATPase. (*ii*) The angle defined by two neighboring antibodies bound to one CF_1 particle is characteristically 120°. These results suggest a symmetrical arrangement of three α subunits in the CF_1 enzyme complex, the overall subunit stoichiometry of which is thus confirmed to be $\alpha_3\beta_3\gamma\delta\epsilon$. Most likely, this type of subunit organization has evolved as a general structural principle for ATPases of the F_1 type.

For some time, however, an F_1 subunit structure as described above appeared to be in conflict with results from single-crystal x-ray analysis. This technique, most powerful in terms of resolution, has been successfully applied to only one ATPase of the F_1 type: the one from rat liver mitochondria (30, 31). From a 0.9-nm-resolution electron density map, Amzel *et al.* (31) concluded that the enzyme may be composed of two equivalent half structures related by a 2-fold axis of symmetry (31). As pointed out in ref. 4, these results suggest a dimeric stoichiometry of the kind $(\alpha\beta\gamma\delta\epsilon)_2$, while they seem to be compatible with a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ only if one postulates that neither of the major

subunits (α and β) exists in the complex as a symmetrical trimer. This view is in conflict with our results, however. Although we do not claim that all three α subunits are structurally or functionally equivalent at all times, the pattern of antibody binding suggests that the positions occupied by the α subunits in the CF_1 complex are indeed related by 3-fold symmetry.

Nonequivalence of α subunits is actually indicated by the cyclic type of immune complexes (Fig. 3*d*). Under all experimental conditions applied, this kind of immune complex was the most frequent one. This observation directly corroborates a recent hypothesis that postulates cyclic immune complexes to be the cause of enhanced antibody avidities (32, 33). Surprising, however, is the fact that the reaction sequence is interrupted at this point: quite often, one of three α subunits fails to make permanent contact to a mAb so that cyclic complexes in which all six α-subunits were occupied by IgG molecules were observed rarely only. We attribute this phenomenon to the considerable torsional stress exerted by two antibodies on two CF_1 ATPase molecules in the rather tense structure of a cyclic immune complex. This stress may induce a conformational change in the enzyme resulting in a reduced antigenicity of the third α subunit. Thus, by anticooperative interactions, binding of two antibodies to a CF_1 ATPase molecule in a cyclic complex would decrease the affinity constant for binding of a third antibody. Of course, other reasons—such as *a priori* different α subunits or nonequivalent contacts to the minor subunits γ, δ, and ε—cannot be ruled out; they appear less likely, however, because on the other hand the reaction easily proceeds to saturation (three anti-α-subunit IgGs per CF_1 ATPase molecule) as long as immune complexes contain one CF_1 ATPase molecule only. In addition, the observation that all three α-subunits of a CF_1 ATPase molecule are occasionally occupied by antibodies in cyclic complexes (Fig. 3*d*) points to an induced asymmetry rather than to a permanent one.

A frequent occurrence of cyclic immune complexes would have led to erroneous results had not the subunit stoichiometry been determined by direct visualization of all types of immune complexes. As the IgG/CF_1 ATPase ratio in cyclic complexes is 1 for those examples shown in Fig. 3*d* (and 2 for the rarely occurring fully saturated ones), any technique evaluating antigen–antibody interactions on an average would have yielded stoichiometric numbers ranging between 1 and 3. In fact, in a report on the subunit stoichiometry of the F_1 ATPase from pig heart mitochondria, Moradi-Ameli and Godinot (34) described radioimmunoassays with subunit-specific mAbs in which average stoichiometric numbers were found of 1.3, 1.4, 2.2, and 2.5, respectively, for four different mAbs. The discrepancy between these numbers and the actual stoichiometric value of 3 (in ref. 34, it is assumed that three α and three β subunits are present in the enzyme) can be explained most easily by the diversity of immune complexes that has been visualized in our study.

We thank Drs. K. Ehrig and P. Friedl (Braunschweig) for stimulating discussions. This work would not have been possible without the superb technical assistance of C. Bergmann (Braunschweig), A. Lassen (Lübeck), and C. Lubitz (Lübeck) nor without a generous grant from the Deutsche Forschungsgemeinschaft.

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